Heterogeneity of natural Foxp3$^+$ T cells: A committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity

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Natural regulatory T cells (T_{reg}) represent a distinct lineage of T lymphocytes committed to suppressive functions, and expression of the transcription factor Foxp3 is thought to identify this lineage specifically. Here we report that, whereas the majority of natural CD4$^+$Foxp3$^+$ T cells maintain stable Foxp3 expression after adoptive transfer to lymphopenic or lymphoreplete recipients, a minor fraction enriched within the CD25$^-$ subset actually lose it. Some of those Foxp3$^-$ T cells adopt effector helper T cell (T_h) functions, whereas some retain "memory" of previous Foxp3 expression, reacquiring Foxp3 upon activation. This minority "unstable" population exhibits flexible responses to cytokine signals, relying on transforming growth factor-β to maintain Foxp3 expression and responding to other cytokines by differentiating into effector T_h in vitro. In contrast, CD4$^+$Foxp3$^+$CD25^{high} T cells are resistant to such conversion to effector T_h even after many rounds of cell division. These results demonstrate that natural Foxp3$^+$ T cells are a heterogeneous population consisting of a committed T_{reg} lineage and an uncommitted subpopulation with developmental plasticity.

immunological tolerance | lineage commitment | transcription factor

Compelling evidence indicates that natural T_{reg}, initially identified as CD4$^+$CD25$^+$ T cells, are central to the establishment and maintenance of immunological self-tolerance and immune homeostasis (1). Such cells express predominantly, if not specifically, the transcription factor Foxp3 (2–4). Studies in mice engineered to express a Foxp3 reporter confirmed that Foxp3 expression is confined to a subset of $\alpha\beta$ T cells and correlates with suppressor activity irrespective of CD25 expression (5, 6). Furthermore, mutations of the Foxp3 gene result in the development of a catastrophic lymphoproliferative autoimmune disorder in mice and humans due to defective generation of functional T_{reg} (3, 4), whereas ectopic Foxp3 expression in conventional CD4$^+$ T cells confers a phenotype and function similar to, if not identical to, natural T_{reg} (2–4). More recently, continuous and high levels Foxp3 expression was found necessary for maintenance of T_{reg} phenotype and function (7, 8). These findings collectively led to the notion that Foxp3 represents a specific and faithful molecular marker to identify the T_{reg} lineage and acts as its "specification factor" or "master regulator" (2, 5).

Early studies of autoimmunity provoked by neonatal thymectomy in mice (1), and of transplantation tolerance to tissue grafts induced by previous transplantation of pure thymic epithelium in birds and in mice (9), indicated that natural T_{reg} represent a distinct thymus-committed lineage. Although it has also become evident that further Foxp3$^+$ T_{reg} can be generated from peripheral naïve CD4$^+$ T cells upon "tolerogenic" antigen presentation in vivo or activation in the presence of transforming growth factor (TGF)-β *in vitro* (10, 11), this notion of a stable lineage has been strengthened by data showing that the phenotypic and functional features of CD4$^+$CD25$^+$ T_{reg} are stably maintained after many rounds of cell division in vitro and in vivo (1). In line with this, the majority, if not all, of CD4$^+$CD25$^+$ T cells appear to maintain Foxp3 expression when transferred into normal lymphoreplete mice, and their Foxp3 stability has been associated with chromatin remodeling of the Foxp3 locus (12). All these studies examined phenotypic and functional stability of CD4$^+$CD25$^+$ T cells at the population level, leaving it unclear as to whether absolutely all natural Foxp3$^+$ T cells were so fixed in their behavior.

In contrast, others have claimed that Foxp3 expression may not be entirely specific for the T_{reg} lineage and that natural T_{reg} may have a much more plastic phenotype. First, human naïve CD4$^+$ T cells were shown to up-regulate FOXP3 transiently at low levels after in vitro TCR stimulation without acquiring T_{reg} characteristics (13). Second, Foxp3 expression in murine TGF-β-induced Foxp3$^+$ T cells (inducible T_{reg} or iT_{reg}) has been claimed to be unstable and to be readily lost upon secondary stimulation (12). Third, some natural Foxp3$^+$ T cells from Foxp3 reporter mice were recently shown to down-regulate Foxp3 and "transdifferentiate" into interleukin (IL)-17-producing effector T_h under the influence of IL-6 and autocrine TGF-β (14, 15). These observations clearly challenge the prevailing notion, yet the root of this apparent contradiction is unclear.

This study was undertaken to evaluate the stability of Foxp3 expression and T_{reg} phenotype of CD4$^+$Foxp3$^+$ T cells isolated from nonmanipulated normal mice in vivo and in vitro. We demonstrate heterogeneity within natural Foxp3$^+$ T cells and provide evidence that they comprise a majority committed T_{reg} lineage and a minor subpopulation with developmental plasticity.

Results

Some Peripheral CD4$^+$Foxp3$^+$ T Cells Lose Foxp3 Expression Under Lymphopenic and Lymphoreplete Conditions. CD4$^+$EGFP$^+$ and EGFP$^-$ T cells were sorted from the spleen and LN of Foxp3^{EGFP} mice and transferred into RAG2$^{-/-}$ mice either alone or mixed at a 1:1 or 1:10 ratio. To distinguish the two donor populations in the co-transferred group, EGFP$^+$ and EGFP$^-$ cells were obtained from Foxp3^{EGFP} Ly5.2 and Ly5.1 congenic mice, respectively. Four weeks after transfer, LN (Fig. 1*A*) and spleen (not depicted) were analyzed for EGFP expression in donor CD4$^+$ T cells. Approximately 50% of donor CD4$^+$ T cells were EGFP$^-$ in the recipients of EGFP$^+$ cells alone. The generation of EGFP$^-$ cells from EGFP$^+$

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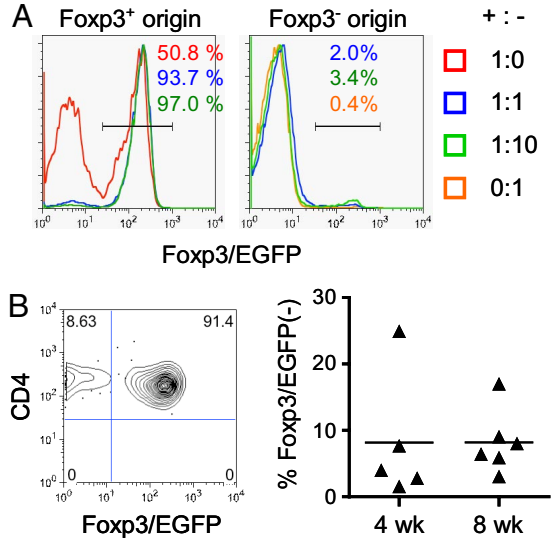

Fig. 1. A fraction of CD4$^+$Foxp3$^+$ T cells lose Foxp3 expression in lymphopenic and normal lymphoreplete conditions. (*A*) CD4$^+$EGFP$^+$ and EGFP$^-$ T cells were sorted from Foxp3^{EGFP} Ly5.2 and Foxp3^{EGFP} Ly5.1 mice, respectively, and adoptively transferred into RAG2$^{-/-}$ mice, either alone (1×10^5 each) or mixed at a 1:1 or 1:10 ratio (1×10^5 EGFP$^+$ plus 1×10^5 or 1×10^6 EGFP$^-$). Four weeks after transfer, LN cells were stained for CD4, TCRβ and Ly5.1. Shown are representative EGFP expression profiles of the Foxp3$^+$ (Ly5.1$^-$) or Foxp3$^-$ (Ly5.1$^+$) donor-derived CD4$^+$TCRβ^+ cells recovered from the indicated host mice. (*B*) 1×10^5 CD4$^+$EGFP$^+$ T cells sorted from Foxp3^{EGFP} Ly5.1/Thy1.2 mice were adoptively transferred into Ly5.2/Thy1.1 host mice 4 and 8 weeks earlier. Pooled LN and spleen cells were first enriched for donor T cells by depleting Thy1.1$^+$, Ig$^+$, and adherent cells by panning, and were stained for Ly5.1, Thy1.2, and CD4. A representative EGFP and CD4 profile of the Ly5.1$^+$Thy1.2$^+$ donor cells (left) and frequencies of EGFP$^-$ cells in CD4$^+$Ly5.1$^+$Thy1.2$^+$ donor T cells (right) are shown. Each symbol indicates individual host mouse.

cells was not caused by EGFP$^-$ contaminants in the inocula but reflected Foxp3 down-regulation, as small numbers of Ly5.1 EGFP$^-$ T cells that had been mixed into the sorted Ly5.2 EGFP$^+$ T cells (manipulated such that similar numbers of Ly5.1 and Ly5.2 EGFP$^-$ cells were injected) failed to "grow out" within the recipients [supporting information (SI) Fig. S1]. When co-transferred with EGFP$^-$ cells, the numbers of EGFP$^+$ donor cells that maintained EGFP expression (hereafter denoted as Foxp3$^{+\rightarrow+}$ T cells) increased in a manner depending on the numbers of co-injected EGFP$^-$ cells (Fig. 1*A*, Fig. S2*A*), indicating that Foxp3$^-$ cells promoted expansion and/or survival of Foxp3$^+$ cells. In contrast, the numbers of EGFP$^+$ donor cells that had lost EGFP expression (Foxp3$^{+\rightarrow-}$ T cells) were reduced when co-transferred with EGFP$^-$ T cells, indicating that Foxp3$^-$ cells inhibited the expansion/survival of Foxp3$^{+\rightarrow-}$ T cells or the down-regulation of Foxp3 expression. The loss of Foxp3 expression and its inhibition by Foxp3$^-$ T cells was also observed when CD3$\varepsilon^{-/-}$ mice were used as hosts, indicating that the presence of B cells does not affect Foxp3 down-regulation (Fig. S2*B*). As higher numbers of donor T cells could be obtained from CD3$\varepsilon^{-/-}$ hosts compared with RAG2$^{-/-}$ hosts, CD3$\varepsilon^{-/-}$ mice were used in some of the following analyses when large numbers of donor-derived T cells were needed.

To determine whether Foxp3 down-regulation from CD4$^+$Foxp3$^+$ T cells takes place under lymphoreplete conditions, we adoptively transferred CD4$^+$EGFP$^+$ T cells sorted from Foxp3^{EGFP} Ly5.1/Thy1.2 mice into Ly5.2/Thy1.1 mice. Use of the dual congenic markers as well as pre-enrichment of donor cells allowed us to faithfully detect small numbers of donor T cells as Ly5.1$^+$Thy1.2$^+$ cells. Approximately 8% of EGFP$^+$ donor T cells were EGFP$^-$ when analyzed at 4 and 8 weeks after transfer (Fig. 1*B*).

These results demonstrate that some Foxp3$^+$ T cells had down-regulated Foxp3 expression in both lymphopenic and lymphoreplete hosts, suggesting that Foxp3 down-regulation represents a normal physiological process. Inasmuch as the frequencies of Foxp3$^{+\rightarrow-}$ T cells were low in the presence of Foxp3$^-$ "competitor" T cells, they must normally be a somewhat rare population.

The Majority of Foxp3-Down-Regulated T Cells Lose T_{reg} Phenotype and Function and Produce Inflammatory Cytokines. We next examined whether CD4$^+$Foxp3$^+$ T cells lose T_{reg} phenotype and function upon loss of Foxp3 expression and, if so, whether they differentiate into effector T_h. CD25, GITR, and CTLA-4, well-known T_{reg} phenotypic markers were less abundant on Foxp3$^{+\rightarrow-}$ T cells than on Foxp3$^{+\rightarrow+}$ T cells, and comparable to control Foxp3$^-$ T cells recovered from RAG2$^{-/-}$ recipients of Foxp3$^-$ T cells alone (Foxp3$^{-\rightarrow-}$ T cells) (Fig. 2*A*). When stimulated in vitro with anti-CD3, both Foxp3$^{+\rightarrow+}$ T cells as well as EGFP$^+$ T cells from Foxp3^{EGFP} mice failed to proliferate, indicating, as expected, that Foxp3$^{+\rightarrow+}$ T cells maintained their anergic state (Fig. 2*B*). In contrast, Foxp3$^{+\rightarrow-}$ T cells proliferated to a greater extent than Foxp3$^{+\rightarrow+}$ T cells, yet much less than EGFP$^-$ T cells from Foxp3^{EGFP} mice, indicating a partial anergy (Fig. 2*B*). When co-cultured with CD4$^+$CD25$^-$ responder T cells, Foxp3$^{+\rightarrow-}$ T cells did not suppress their proliferation as efficiently as Foxp3$^{+\rightarrow+}$ T cells (Fig. 2*C*). However, responder T cells proliferated slightly less in the presence of Foxp3$^{+\rightarrow-}$ T cells than in their absence, whereas Foxp3$^{-\rightarrow-}$ T cells enhanced responder T-cell proliferation. These results indicate that Foxp3$^{+\rightarrow-}$ T cells had lost their suppressive activity, although not completely.

To examine whether Foxp3$^{+\rightarrow-}$ T cells had differentiated into effector T_h in lymphopenic hosts, Foxp3$^{+\rightarrow+}$, Foxp3$^{+\rightarrow-}$, and Foxp3$^{-\rightarrow-}$ T cells were stimulated ex vivo and analyzed for their cytokine production at the single cell level (Fig. 2*D*). Many Foxp3$^{+\rightarrow-}$ T cells produced significant IL-2, IL-17, and IFN-γ compared with Foxp3$^{+\rightarrow+}$ T cells. Notably, Foxp3$^{+\rightarrow-}$ T cells preferentially produced IL-2 or IL-17 compared with Foxp3$^{-\rightarrow-}$ T cells, which mainly produced IFN-γ. None of these populations produced IL-4 at a significant frequency (<1%) (not depicted). These results suggest that at least some Foxp3$^+$ T cells had differentiated into effector T_h.

A Significant Fraction of Foxp3-Down-Regulated T cells Reacquire Foxp3 Expression in Vitro and in Vivo. In the above in vitro suppression assay, we noted that a fraction of Foxp3$^{+\rightarrow-}$ T cells re-expressed EGFP, whereas Foxp3$^{-\rightarrow-}$ T cells or Foxp3$^-$ T cells from Foxp3^{EGFP} mice did not (not shown). This preferential Foxp3 re-induction from Foxp3$^{+\rightarrow-}$ T cells was also seen when stimulated with anti-CD3/CD28 mAbs-coated beads (Fig. 3*A*). Foxp3$^{+\rightarrow-}$ T cells re-expressed Foxp3 in a TGF-β-independent manner, as neutralizing concentrations of anti-TGF-β mAbs and TGFβRII-Fc chimeric proteins did not affect the outcome (Fig. 3*B*). In contrast, TCR stimulation was essential, because Foxp3$^{+\rightarrow-}$ T cells failed to reacquire EGFP expression without anti-CD3/CD28 beads (not shown). The observed re-induction of Foxp3 expression in a fraction of Foxp3$^{+\rightarrow-}$ T cells may well explain their partially anergic phenotype and weak suppressor activity (Fig. 2 *B* and *C*) despite a large number of IL-2-producing cells (Fig. 2*D*).

To address whether Foxp3$^{+\rightarrow-}$ T cells preferentially reacquire Foxp3 expression in vivo, we injected Foxp3$^{+\rightarrow-}$ T cells or control Foxp3$^{-\rightarrow-}$ T cells from CD3$\varepsilon^{-/-}$ hosts into secondary CD3$\varepsilon^{-/-}$ hosts. We found that ≈10% of Foxp3$^{+\rightarrow-}$ T cells re-expressed EGFP, whereas few Foxp3$^{-\rightarrow-}$ T cells and only ≈1% of naïve EGFP$^-$ T cells up-regulated EGFP (Fig. 3*C*).

These results indicate that Foxp3$^{+\rightarrow-}$ T cells are heterogeneous and comprise not only effector T_h but also putative T_{reg} precursors poised for Foxp3 transcription.

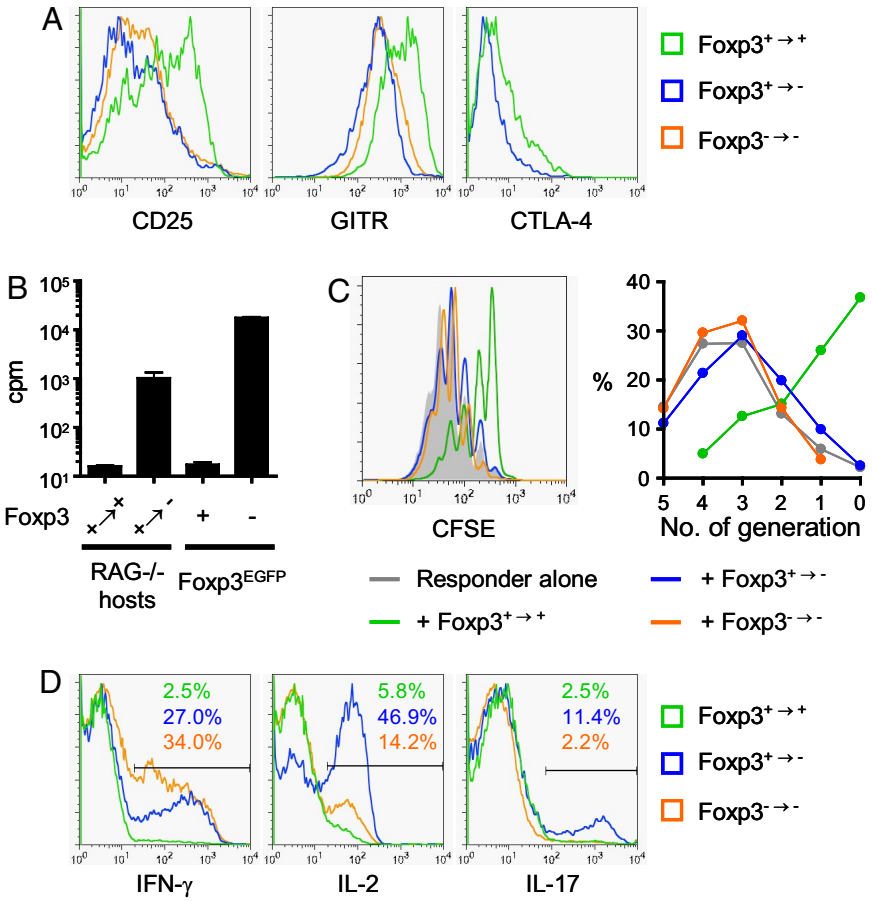

Fig. 2. CD4$^+$Foxp3$^+$ T cells lose T_{reg} phenotype and produce inflammatory cytokines upon down-regulation of Foxp3. (*A*) CD4$^+$EGFP$^+$ or EGFP$^-$ T cells were transferred into RAG2$^{-/-}$ mice as in Fig. 1A. Four weeks later, LN cells were stained for TCRβ, CD4, and the indicated markers. Shown are representative profiles of CD25, GITR, and CTLA-4 expression on TCRβ^+CD4$^+$EGFP$^+$ (Foxp3$^{+\to+}$) and EGFP$^-$ (Foxp3$^{+\to-}$) cells from the recipients of EGFP$^+$ cells and on TCRβ^+CD4$^+$EGFP$^-$ cells from the recipients of EGFP$^-$ cells (Foxp3$^{-\to-}$). (*B–D*) CD4$^+$EGFP$^+$ or EGFP$^-$ T cells from Foxp3^{EGFP} Ly5.1 mice were transferred into RAG2$^{-/-}$ (*B*, *C*) or CD3$\varepsilon^{-/-}$ (*D*) mice 4 weeks earlier. Pooled LN and spleen cells were stained for CD4, and Ly5.1, and Foxp3$^{+\to+}$, Foxp3$^{+\to-}$, and Foxp3$^{-\to-}$ CD4$^+$Ly5.1$^+$ donor cells were sorted. (*B*) Indicated populations were stimulated in vitro with anti-CD3, and proliferation was measured by [^{3}H]thymidine incorporation. (*C*) CFSE-labeled CD4$^+$CD25$^-$ responder T cells from B6 (Ly5.2) mice were stimulated in vitro either alone or together with an equal number of the indicated populations. On day 3, cells were stained for Ly5.1 and CD4, and CFSE dilution on the CD4$^+$Ly5.1$^-$ responder population was determined. Shown are representative CFSE profiles of responder T cells from each group (*left*) and frequencies of cells of the indicated generation (*right*). (*D*) Indicated populations were stimulated with PMA and ionomycin and production of IFN-γ, IL-2, and IL-17 was assessed by intracellular staining.

Foxp3-Down-Regulated T Cells Are Generated by Extensive Lymphopenia-Driven Expansion of a Rare Subpopulation. We observed that Foxp3$^{+\to-}$ T cells constituted ≈50% of Foxp3$^+$ donor-derived cells in lymphopenic mice, whereas they represented a minor population when co-transferred with Foxp3$^-$ T cells or when transferred into normal mice. We wondered whether the "unstable" subpopulation was better able to expand in lymphopenic conditions when competitor Foxp3$^-$ T cells were absent. We therefore examined the relationship between Foxp3 down-regulation and cell division using a Foxp3^{hCD2} reporter that we have established recently. Sorted CD4$^+$hCD2$^+$ T cells were labeled with CFSE, injected into RAG2$^{-/-}$ mice, and analyzed for hCD2 expression simultaneously with CFSE dilution on days 5, 7, and 14 after transfer. On day 5, only ≈2% of donor cells were hCD2$^-$; but ≈83% of this small number of Foxp3$^{+\to-}$ T cells had already divided more than once whereas ≈60% of Foxp3$^{+\to+}$ T cells still remained undivided, indicating enrichment of proliferating cells in Foxp3$^{+\to-}$ T cells (Fig. 4). The frequencies of hCD2$^-$ T cells progressively increased through days 7 to 14, and all of the

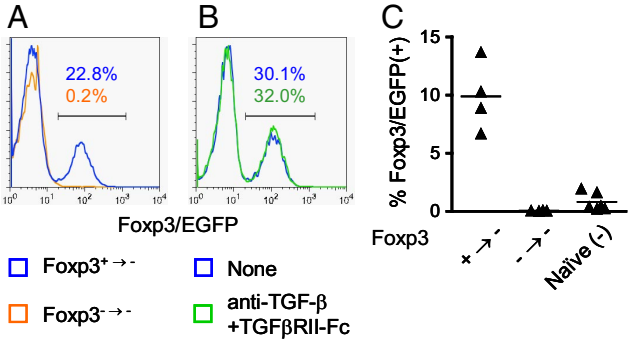

Fig. 3. Preferential re-induction of Foxp3 expression in Foxp3$^-$down-regulated T-cells in vitro and in vivo. CD4$^+$Foxp3$^{+\to-}$ and Foxp3$^{-\to-}$ donor T cells were sorted from CD3$\varepsilon^{-/-}$ hosts as described in Fig. 2B-D. (*A*) Sorted cells were stimulated in vitro with anti-CD3→CD28 beads, and analyzed for CD4 and EGFP expression on day 3. (*B*) Foxp3$^{+\to-}$ T cells were stimulated as in A with or without a mixture of anti-TGF-β mAbs and TGFβRII-Fc. (*C*) 1 × 10^5 Foxp3$^{+\to-}$ or Foxp3$^{-\to-}$ T cells or EGFP$^-$ T cells from Foxp3^{EGFP} mice were transferred into new CD3$\varepsilon^{-/-}$ host mice. Four weeks after the transfer, LN and spleen (not depicted) cells were stained for CD4 and Ly5.1, and analyzed for EGFP expression. Shown are frequencies of (re-)induced Foxp3$^+$ T cells in CD4$^+$Ly5.1$^+$ donor T cells. Each symbol represents individual host mouse.

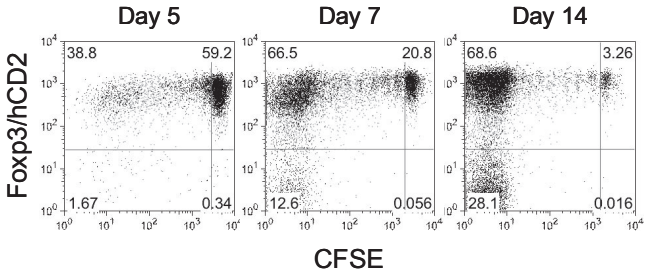

Fig. 4. Foxp3-down-regulated T-cells have proliferated extensively in lymphopenic mice. CD4$^+$hCD2$^+$ T cells (4 × 10^5) from Foxp3^{hCD2} Ly5.1 mice were labeled with CFSE and injected into RAG2$^{-/-}$ mice. On the indicated time points, LN and spleen cells from all recipient mice (n = 3) were pooled and stained for hCD2, Ly5.1, and Ly5.2. Shown are hCD2 and CFSE profiles on Ly5.1$^+$Ly5.2$^-$ donor cells.

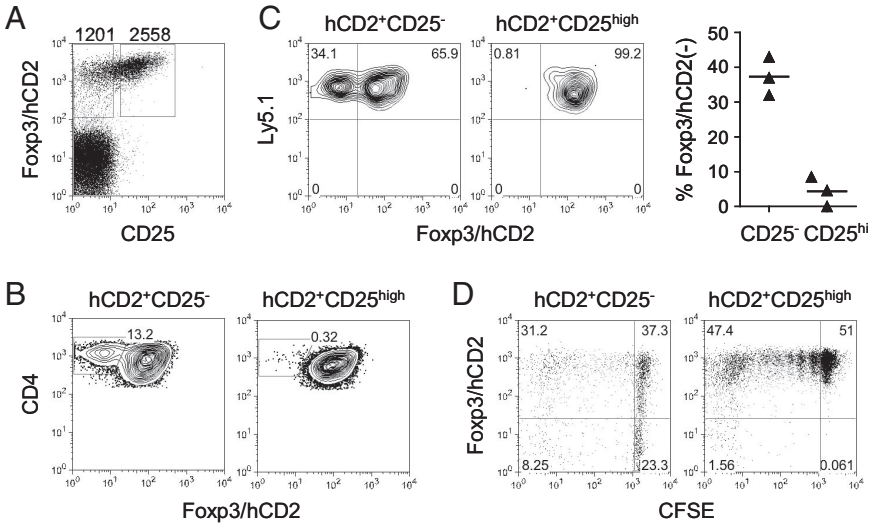

Fig. 5. $CD4^+Foxp3^+CD25^-$ T cells exhibit lower and less stable Foxp3 expression than $CD4^+Foxp3^+CD25^{high}$ T cells. (*A*) LN cells from $Foxp3^{hCD2}$ mice were stained for hCD2, CD4, and CD25. Representative CD25 and hCD2 expression profiles on $CD4^+$ cells are shown. The number above each gate indicates the mean fluorescent intensity of hCD2. (*B*) Sorted $CD4^+hCD2^+CD25^-$ and $CD25^{high}$ T cells were stimulated in vitro with anti-CD3/CD28 beads in the presence of IL-2 and analyzed for hCD2 and CD4 expression on day 3. (*C*) 1×10^5 Ly5.1 $CD4^+hCD2^+CD25^-$ and $CD25^{high}$ T cells were transferred into $RAG2^{-/-}$ mice, and LN cells were stained for hCD2, Ly5.1, and Ly5.2 on day 5. Shown are representative hCD2 and Ly5.1 expression profiles (*left*) and frequencies of $hCD2^-$ cells (*right*) in donor ($Ly5.1^+Ly5.2^-$) cells. (*D*) Ly5.1 $CD4^+hCD2^+CD25^-$ and $CD25^{high}$ T cells were stained with CFSE and transferred into $Foxp3^{hCD2}$ Ly5.2 mice (4×10^5 cells). On day 14, pooled LN and spleen cells were stained for Ly5.1, Ly5.2, and hCD2 and enriched for donor $Ly5.1^+$ cells by magnetic sorting before analysis. Representative CFSE and hCD2 profiles on $Ly5.1^+Ly5.2^-$ cells are shown.

$Foxp3^{+\rightarrow-}$ T cells had lost CFSE, indicating that they had undergone multiple rounds of cell division. These results suggest that the $Foxp3^{+\rightarrow-}$ T cells represent progeny of a relatively rare subpopulation of $Foxp3^+$ T cells that undergo extensive expansion in lymphopenic conditions. However, it remains possible that any $Foxp3^+$ T cells can lose Foxp3 expression with a certain probability, requiring a defined number of divisions to lose Foxp3.

To distinguish these two possibilities, we compared the TCR repertoires of $Foxp3^{+\rightarrow+}$ and $Foxp3^{+\rightarrow-}$ T cells by flow cytometry using mAbs for Vα2 and 6 Vβ chains. If only a rare subpopulation of $Foxp3^+$ T cells lost Foxp3 and then expanded, their TCR repertoires would be more oligoclonal than $Foxp3^{+\rightarrow+}$ T cells and the two repertoires should largely be distinct. In contrast, if any $Foxp3^+$ T cells can down-regulate Foxp3 after a defined number of cell divisions, the two repertoires ought to be similar to each other. As shown in Fig. S3, although the patterns of Vα/Vβ usage in polyclonal $Foxp3^+$ T cells were stable among different individual mice, those in both $Foxp3^{+\rightarrow+}$ T cells and $Foxp3^{+\rightarrow-}$ T cells were highly variable, indicative of oligoclonal expansion. However, the variation in $Foxp3^{+\rightarrow-}$ T cells was significantly larger than in $Foxp3^{+\rightarrow+}$ T cells, suggesting the former to be more oligoclonal than the latter. Moreover, within each individual mouse, $Foxp3^{+\rightarrow-}$ T cells and $Foxp3^{+\rightarrow+}$ T cells showed distinct Vα/Vβ usage patterns, suggesting that clones expanding in the two populations were different. These results support the notion that $Foxp3^{+\rightarrow-}$ T cells represent progeny of a relatively rare subpopulation of $Foxp3^+$ T cells.

$CD4^+Foxp3^+CD25^-$ T Cells Exhibit Unstable, Whereas $CD4^+Foxp3^+CD25^+$ T Cells Exhibit Stable, Foxp3 Expression. Let us suppose that $Foxp3^+$ T cells in the normal T-cell repertoire harbor a minor population of unstable $Foxp3^+$ T cells. How might these be distinguished from stable $Foxp3^+$ T cells? We asked whether Foxp3 stability could be associated with the expression of a Foxp3-dependent marker. We found that $CD4^+Foxp3^+CD25^-$ T cells expressed lower levels of the hCD2 reporter than did $CD4^+Foxp3^+CD25^{high}$ T cells (Fig. 5*A*). The former subset also expressed GITR and CTLA-4 at lower levels than the latter (not shown). In line with their lower Foxp3 expression, $CD4^+Foxp3^+CD25^-$ T cells were less anergic and suppressive than $CD4^+Foxp3^+CD25^{high}$ T cells (Fig. S4). These results led us to hypothesize that $CD4^+Foxp3^+CD25^-$ T cells might be the ones harboring uncommitted T_{reg}, the Foxp3 expression of which is labile.

We sorted $CD4^+Foxp3^+$ T cells into $CD25^-$ and $CD25^{high}$ cells and tested their Foxp3 stability in vitro and in vivo. $Foxp3^{hCD2}$ mice were used instead of $Foxp3^{EGFP}$ mice in these analyses, because the hCD2 reporter enables better separation of $Foxp3^{low}$ cells from $Foxp3^-$ cells due to its high sensitivity. In addition, pre-enrichment of $hCD2^+$ cells by magnetic sorting followed by FACS sorting yielded a highly pure (>99%) population of this small subset. When $hCD2^+CD25^-$ and $CD25^{high}$ T cells were stimulated in vitro with anti-CD3/CD28 beads in the presence of IL-2, we found that ≈11% of the former population lost hCD2 expression whereas >99% of the latter maintained hCD2 (Fig. 5*B*). Furthermore, when transferred into $RAG2^{-/-}$ mice, ≈37% of $Foxp3^+CD25^-$ T cells preferentially down-regulated hCD2, whereas most of $Foxp3^+CD25^{high}$ T cells maintained hCD2 (Fig. 5*C*).

When labeled with CFSE and transferred into $Foxp3^{hCD2}$ Ly5.2 mice, the vast majority of $hCD2^+CD25^{high}$ T cells maintained hCD2 expression even after multiple cycles of cell division, although 1–2% of them down-regulated hCD2 while losing CFSE (Fig. 5*D*). In contrast, ≈32% of $hCD2^+CD25^-$ T cells lost hCD2 expression, the majority (≈75%) of which did so without cell division. This lack of cell division of $Foxp3^{+\rightarrow-}$ T cells accounts for their low representation in nonlymphopenic conditions and in turn supports the idea that they are generated by extensive proliferation of a rare subset of $Foxp3^+$ T cells in lymphopenic conditions.

These results collectively indicate that the unstable cells come from a rare subpopulation enriched within the $CD25^-$ subset of $Foxp3^+$ T cells.

$CD4^+Foxp3^+CD25^-$ T Cells Contain Uncommitted T_{reg} That Retain T_h Potential, Whereas $CD4^+Foxp3^+CD25^{high}$ T Cells Represent a Committed T_{reg} Lineage. Our results suggest that some of $CD4^+Foxp3^+CD25^-$ T cells remain uncommitted to T_{reg} fate and retain options on alternative T_h pathways, whereas $CD4^+Foxp3^+CD25^{high}$ T cells represent a committed T_{reg} lineage. Developmental plasticity of uncommitted progenitors can be revealed when they are placed in environments that are permissive for alternative fate choices. It is well established that cytokines provide such "environments." TGF-β is known to induce Foxp3 expression and to instruct "iT_{reg}" differentiation, whereas IL-12, IL-4, and IL-6 together with TGF-β promote T_h1, T_h2, and T_h17 differentiation, respectively. We asked whether $CD4^+Foxp3^+CD25^-$ and $CD25^{high}$ T cells depended on TGF-β for their Foxp3 maintenance and whether the above cytokines would encourage differentiation into effector T_h subsets.

$CD4^+Foxp3^+CD25^-$ and $CD25^{high}$ T cells were stimulated with anti-CD3/CD28 beads in the presence or absence of TGF-β1 or anti-TGF-β mAbs (Fig. 6*A*). The proportion of $Foxp3^+CD25^-$ T cells that lost hCD2 expression increased 3-fold with stimulation in the presence of anti-TGF-β, whereas they not only maintained but also up-regulated hCD2 in the presence of TGF-β1. In contrast, few

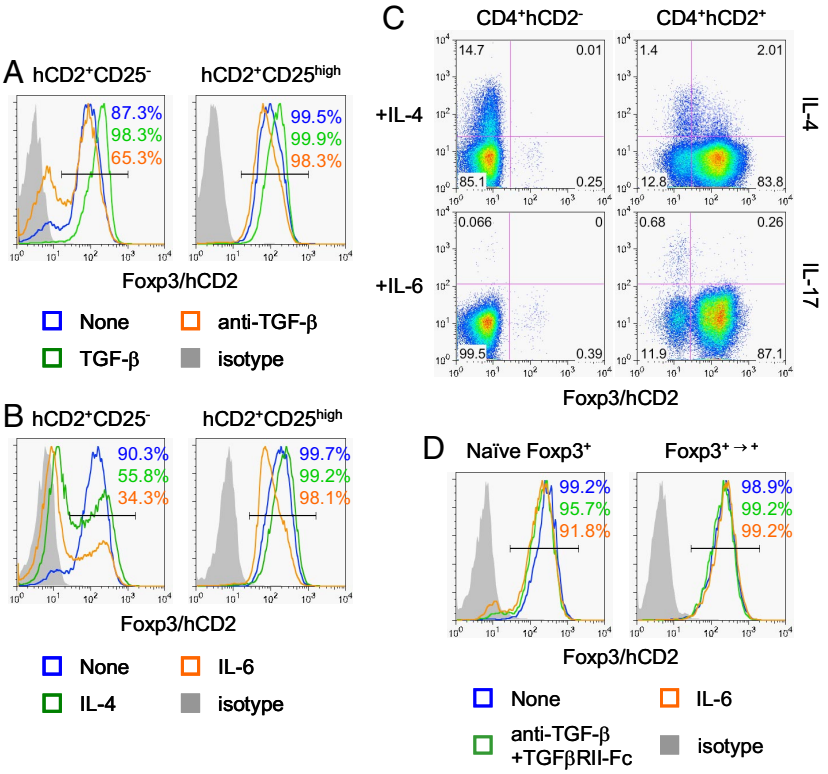

Fig. 6. Only CD4$^+$Foxp3$^+$CD25$^-$ T cells require TGF-β for Foxp3 maintenance. These cells can be instructed by cytokines to differentiate into effector T$_h$, whereas CD4$^+$Foxp3$^+$CD25^{high} T cells cannot. (*A*, *B*) Sorted CD4$^+$hCD2$^+$CD25$^-$ and CD25^{high} T cells were stimulated in vitro with anti-CD3/CD28 beads in the presence of IL-2 with or without the indicated cytokines or mAbs. On day 3, cells were stained with anti-hCD2 or its isotype control mAbs. (*C*) Sorted CD4$^+$hCD2$^+$ and hCD2$^-$ T cells were stimulated in vitro with anti-CD3/CD28 beads in the presence of IL-4 or IL-6. On day 5, cells were re-stimulated and stained for IL-4 or IL-17 production. (*D*) Total Ly5.1 CD4$^+$Foxp3^{hCD2+} T cells were transferred into CD3$\varepsilon^{-/-}$ mice 4 weeks earlier. Sorted CD4$^+$Foxp3$^{+\to+}$ Ly5.1$^+$ and hCD2$^+$ T cells from Foxp3^{hCD2} mice were stimulated in the presence of the indicated reagents.

Foxp3$^+$CD25^{high} T cells lost hCD2 expression when TGF-β was neutralized, whereas their hCD2 expression levels were slightly down-regulated. These results indicate that only Foxp3$^+$CD25$^-$ T cells require the continuous presence of TGF-β to maintain Foxp3 expression, although TGF-β also modulates Foxp3 levels in Foxp3$^+$CD25^{high} T cells.

When stimulated in the presence of IL-4, only Foxp3$^+$CD25$^-$ T cells generated Foxp3$^-$ cells (Fig. 6*B*). Interestingly, Foxp3$^+$CD25^{high} T cells up-regulated Foxp3 expression in response to IL-4, indicating that IL-4 signals exert two opposing effects on Foxp3 expression in the two populations. Furthermore, IL-4 promoted IL-4 production primarily in Foxp3$^{+\to-}$ T cells at frequencies (10%) similar to IL-4$^+$ cells induced in control hCD2$^-$ T cells (14%), whereas few (2%) Foxp3$^{+\to+}$ T cells produced IL-4 (Fig. 6*C*), indicating T$_h$2 differentiation from Foxp3$^{+\to-}$ T cells. Likewise, IL-6 promoted loss of Foxp3 expression only in Foxp3$^+$CD25$^-$ T cells (Fig. 6*B*). In contrast to IL-4, however, Foxp3 expression levels were slightly down-regulated in Foxp3$^+$CD25^{high} T cells when stimulated in the presence of IL-6, indicating that IL-6 signals exert negative effects on Foxp3 expression in both populations. Consistent with previous reports (14, 15), IL-6 promoted IL-17 production only in Foxp3$^{+\to-}$ T cells, indicating that T$_h$17 differentiation requires loss of Foxp3 expression (Fig. 6*C*). Interestingly, IL-12 failed to affect Foxp3 expression in both populations and to induce IFN-γ production, whereas it did promote IFN-γ production in $\approx$66% of Foxp3$^-$ T cells (not shown). Although many Foxp3$^{+\to-}$ T cells produced large amounts of IFN-γ (Fig. 2*D*), this result suggests that IL-12 signaling alone is not sufficient to convert Foxp3$^+$ T cells into T$_h$1.

If Foxp3$^+$ T cells in the normal T-cell repertoire largely comprise a committed T$_{reg}$ lineage, they should be capable of maintaining Foxp3 expression even after multiple rounds of cell division. We therefore examined Foxp3 stability in Foxp3$^{+\to+}$ T cells, which had proliferated extensively in lymphopenic hosts, in response to TGF-β withdrawal or co-stimulation with IL-6 or IL-4. When activated in the presence of IL-6 or anti-TGF-β mAbs plus TGFβRII-Fc, few if any Foxp3$^{+\to+}$ T cells lost hCD2 expression and their hCD2 expression levels were unaffected, although a fraction of "naïve" Foxp3$^+$ T cells lost hCD2 expression and the remaining cells slightly down-regulated their levels (Fig. 6*D*). Likewise, IL-4 failed to affect Foxp3 expression in Foxp3$^{+\to+}$ T cells, whereas it promoted Foxp3 loss in a fraction ($\approx$3%) of naïve Foxp3$^+$ T cells (not shown). These results clearly show that Foxp3$^{+\to+}$ T cells exhibit even more stable Foxp3 expression than naïve Foxp3$^+$ T cells after many rounds of cell division.

Taken together, these results demonstrate that a fraction of CD4$^+$Foxp3$^+$CD25$^-$ T cells remain uncommitted to T$_{reg}$ fate and retain plasticity to differentiate into effector T$_h$ when stimulated with appropriate cytokine signals. In contrast, CD4$^+$Foxp3$^+$CD25^{high} T cells are resistant to such "reprogramming" of T$_{reg}$ differentiation even after extensive proliferation, and thus constitute a committed T$_{reg}$ lineage.

Discussion

It is widely believed that natural Foxp3$^+$ T cells represent a stable lineage of T lymphocytes committed to suppression. Recent studies have challenged this view and have suggested that Foxp3 expression may not be entirely specific for T$_{reg}$ or that natural Foxp3$^+$ T$_{reg}$ may be a plastic population. To reconcile these apparently contradictory views, we here examined the stability of Foxp3 expression and T$_{reg}$ phenotype in natural Foxp3$^+$ T cells, and we report that they represent a heterogeneous population consisting of a committed T$_{reg}$ lineage and of a minor uncommitted subpopulation that retains developmental plasticity.

Our results demonstrate that Foxp3$^+$ T cells isolated from nonmanipulated normal mice harbor some uncommitted cells that preserve T$_h$ potential; when transferred into lymphopenic mice or activated under appropriate polarizing conditions in vitro, they lost their T$_{reg}$ identity and some became effector T$_h$. These results are consistent with a recent finding of loss of T$_{reg}$ function and de-repression of T$_h$ functions in mature T$_{reg}$ following induced deletion of the Foxp3 gene (7). A previous study showed that CD4$^+$

T cells from Foxp3 transgenic mice lose Foxp3 expression and differentiate into effector T_h when activated under polarizing conditions in vitro (16), although it was not clear whether they were derived from $Foxp3^+$ T cells or small numbers of $Foxp3^-$ T cells that were present in those mice. We have extended these observations to natural $Foxp3^+$ T cells from normal mice and demonstrated that they have the potential to differentiate into effector T_h including T_h17 (14, 15). On the other hand, however, our results are incompatible with the view that natural $Foxp3^+$ T cells represent a fully plastic population in that any cell can be converted to effector T_h. Instead, the majority of natural $Foxp3^+$ T cells, particularly the $CD25^+$ subset, exhibited stable Foxp3 expression even after extensive proliferation and were resistant to conversion into effector T_h, so reflecting a committed T_{reg} lineage that has lost alternative T_h options.

We found that Foxp3-down-regulated T cells preferentially reacquire Foxp3 expression upon in vitro activation or secondary transfer, indicating that they retain "memory" of previous Foxp3 expression. This Foxp3 re-induction was TGF-β-independent, suggesting that its underlying mechanism is distinct from that of *de novo* Foxp3 induction in naïve T cells. Chromatin modifications including selective demethylation of CpG motifs have been implicated in epigenetic imprinting of gene expression, as shown for cytokine genes (17). Indeed, $CD4^+CD25^+$ T cells were recently shown to display such modifications at the Foxp3 locus, particularly complete demethylation of one of the enhancers (12). Importantly, TGF-β-induced $Foxp3^+$ T cells and activated human T cells, both of which show unstable Foxp3 expression, displayed little demethylation, suggesting that the demethylated Foxp3 locus represents a specific signature of the committed T_{reg} lineage (12, 18). In light of the present study, we can speculate that these T cells with "Foxp3 memory" may belong to the committed T_{reg} lineage, in which their Foxp3 locus has been remodeled and now "accessible", yet transiently being inactive for transcription. Because TCR stimulation was required for Foxp3 re-induction, we suggest that TCR signals may keep Foxp3 transcription active within such a remodeled locus.

Finally, the findings of this study have important implications with respect to the role of Foxp3 in T_{reg} lineage commitment. Since some $Foxp3^+$ T cells can be converted to effector T_h, Foxp3 alone is clearly insufficient (although necessary) to commit precursor cells to the T_{reg} lineage. Our data thus support the emerging view that the T_{reg} lineage is determined by a higher-order regulation operating upstream of, and in cooperation with, Foxp3 (19, 20). However, the molecular nature of such a regulatory system is yet to be uncovered.

Materials and Methods

Mice. C57BL/6J (B6) mice were obtained from Clea Japan. B6.$Foxp3^{EGFP}$ (21), B6.$Foxp3^{hCD2}$, B6.$RAG2^{-/-}$, B6.$CD3\varepsilon^{-/-}$, B6.Thy1.1, and B6.Ly5.1 congenic mice were bred under specific pathogen-free conditions in our animal facility and used at 5–12 weeks of age. B6.$Foxp3^{hCD2}$ reporter mice were generated by homologous recombination in a B6-derived ES cell line using a targeting construct in which cDNA encoding a human CD2-human CD52 fusion protein along with an intra-ribosomal entry site was inserted into the 3′ untranslated region of the endogenous Foxp3 locus. Details of the generation and characterization of $Foxp3^{hCD2}$ mice will be reported elsewhere. All animal experiments were performed in accordance with approved protocols from the Institutional Animal Care at RIKEN.

Reagents. Purified and conjugated monoclonal antibodies (mAbs) were purchased from BD Bioscience, eBioscience, or R&D Systems, and recombinant proteins were obtained from R&D Systems or Peprotech. (See *SI Materials and Methods* for details.)

Lymphocyte Preparations, Flow Cytometry, Cell Sorting. Single-cell suspensions from spleen and LN were prepared as described previously (22). Flow-cytometric analyses were performed as described (22) using a FACSCalibur instrument (Becton Dickinson). Cell sorting was performed using a FACSAria cell sorter (Becton Dickinson). (See *SI Materials and Methods* for details.) The purity of the sorted populations was invariably >99%.

T-Cell Functional Assays. For proliferation assays, T cells (2×10^4/well) were stimulated with 0.5 μg/ml anti-CD3 in the presence of T cell-depleted, γ-irradiated B6 spleen cells as antigen-presenting cells (8×10^4/well) for 3 days as described (2). Results are shown as mean [^{3}H]thymidine incorporation $\pm$ SD in triplicate cultures. For suppression assays, CFSE-labeled responder T cells were stimulated alone or together with T_{reg} (5×10^4 each/well) for 3 days with 0.5 μg/ml anti-CD3 in the presence of antigen-presenting cells (2×10^5/well) in 96-well U-bottom plates. To examine Foxp3 stability and T_h differentiation, T cells (1×10^5/well) were stimulated with anti-CD3/anti-CD28 mAbs-coated beads (Dynal) at a 1:1 cell:bead ratio in 96-well, flat-bottom plates. Where indicated, IL-2 (10 ng/ml), IL-4 (10 ng/ml), IL-6 (20 ng/ml), IL-12 (10 ng/ml), TGF-β1 (5 ng/ml), anti-TGF-β1/2/3 (30 μg/ml), and/or TGFβRII-Fc (3 μg/ml) were added. To assess cytokine production, cells were stimulated for 4 hours with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of brefeldin A and then fixed, permeabilized, and stained with anti-cytokine or isotype control mAbs as described elsewhere (2).

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